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14. ABSTRACT

Here, we propose to harness the immune system by immunotherapy (IT) alongside conventional radiotherapy (RT) to improve the treatment of men with advanced or recurrent prostate cancer. The overall aim is to determine whether local irradiation of prostate tumors in a preclinical and clinical setting leads to measurable tumor-specific immune responses and whether tumor vaccination can boost these immune responses possibly leading to better tumor control. Survivin is our tumor antigen of choice, because it seems superior to other prostate tumor antigens. We generated stable mouse prostate cancer cell lines (TRAMP C1 and TRAMP C2), that express human HLA-A2.1 and we were able to confirm that these cells express survivin. These are two important steps as this will allow us to examine the responses to human surviving epitopes that are clinically relevant within a transgenic humanized mouse model. Enumeration of circulating survivin-specific CD8+ T lymphocytes in prostate cancer patients using tetramers indicated that many patients have higher than normal numbers of these T cells and that they are increased further upon completion of radiation treatment. Whether or not this is due to increase in antigenic peptide liberation and whether this will translate to tumor regression we don't know. What is clear is that RT does not induce immune tolerance to surviving making IT approaches feasible in combination with RT.

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1. INTRODUCTION

This proposal aims to improve the treatment of men with advanced or recurrent prostate cancer, who so often fail conventional treatment modalities, such as Radiotherapy (RT). Here, we propose that therapies tackling prostate cancer from multiple angles, are more promising in achieving local and systemic control. We believe, that harnessing the immune system by immunotherapy (IT) alongside RT to further eliminate cancer cells, is an attractive alternative to these patients.

The overall aim of this proposal is to determine whether local irradiation of prostate tumors in a preclinical and clinical setting leads to measurable tumor-specific immune responses. We address the issue as to whether tumor vaccination can boost these immune responses possibly leading to better tumor control. Survivin is our tumor antigen of choice, because it seems superior to other prostate tumor antigens in that it is clearly overexpressed in tumors of the prostate, it controls its growth (anti-apoptotic & pro-proliferative), and it is immunogenic in these patients. The preclinical studies in this proposal use TRAMP C1 and TRAMP C2 mouse prostate cell lines, that were originally derived from the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice that spontaneously develop prostate cancer and closely mimics the development of the human disease (1). Tumor bearing mice will receive local RT with or without a dendritic cell-based vaccine. Survivin-specific immune responses in these animals will be monitored using enzyme-linked immunospot assay (ELISPOT), flow-cytometric tetramer staining, T cell proliferation assay and CTL assays. Additionally, the number of $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells in the tumors, lymph nodes and spleens will be enumerated by FACS. Whether or not this translates directly into tumor cell killing will be determined in vivo by observing tumor growth. These animal experiments will allow us to investigate the possibility that local irradiation affects overall anti-tumor immune-responses, something that has been difficult to assess in the clinic. If this proves to be correct, we will determine whether or not the combined application of radiation and dendritic-cell vaccination can increase specific anti-tumor immune responses and whether this effectively translates into tumor rejection. Some of the in vivo studies will include the systemic application of a COX-2 inhibitor (Celecoxib, oral), a proteasome-inhibitor (PS-341, i.v.) or cytokines (GM-CSF, s.c.). These agents are already in the clinic with and without RT. They are relevant to our choice of tumor antigen. Survivin is a proteasome target during interphase but proteasome inhibitors paradoxically decrease survivin expression and in turn induce apoptosis (2). COX-2 inhibition also augments the ubiquitination of survivin, ultimately leading to its proteasome-dependent degradation and feeding it into the endogenous antigen presentation pathway (3). GM-CSF will enhance endogenous survivin expression. The consequences of these intercepting pathways, in terms of the presentation of survivin will add another dimension to our studies on combining RT with IT. Another powerful feature of our study is reflected by the fact that the results of our preclinical studies will be directly comparable to equivalent studies that we will perform on prostate cancer patients undergoing Radiotherapy.

2. BODY

The first aim of this proposal is to determine as to whether local irradiation of prostate tumors in mice alters measurable tumor-specific immune responses. The preclinical studies in this proposal use TRAMP C1 and TRAMP C2 mouse prostate cell lines, that express human HLA-A2.1 (TRAMP C/2.1) and grow them s.c. in transgenic humanized C57BL/6 (C57Bl/6-K^b2.1), that express the chimeric mouse/human class I MHC, which will allow us to examine the responses to human survivin epitopes that are clinically relevant.

Initial experiments focused on the generation of stable TRAMP C1 and TRAMP C2 cell lines that have been transfected to express HLA2.1. We used a recently developed Lentivirus introducing the HLA2.1 gene (kindly provided by Drs T Ribas and R Koya, UCLA) to establish stable TRAMP C/2.1 cell lines.

TRAMP C2 cells were treated by spin-inoculation twice on two consecutive days and left to recover for 3 days. Antibody-staining for the human HLA2.1 antigen (clone BB7.2, BD Pharmingen) and FACS analysis revealed low expression levels with only 15-20% truly positive cells (Figure 1A). Because this Lentivirus does not contain a selection marker to prevent possible anti-viral immune responses in vivo, we selected for HLA2.1-positive cells by cloning. 100 cells in single-cell suspensions were plated in 10cm dishes. 5 out of 20 clones

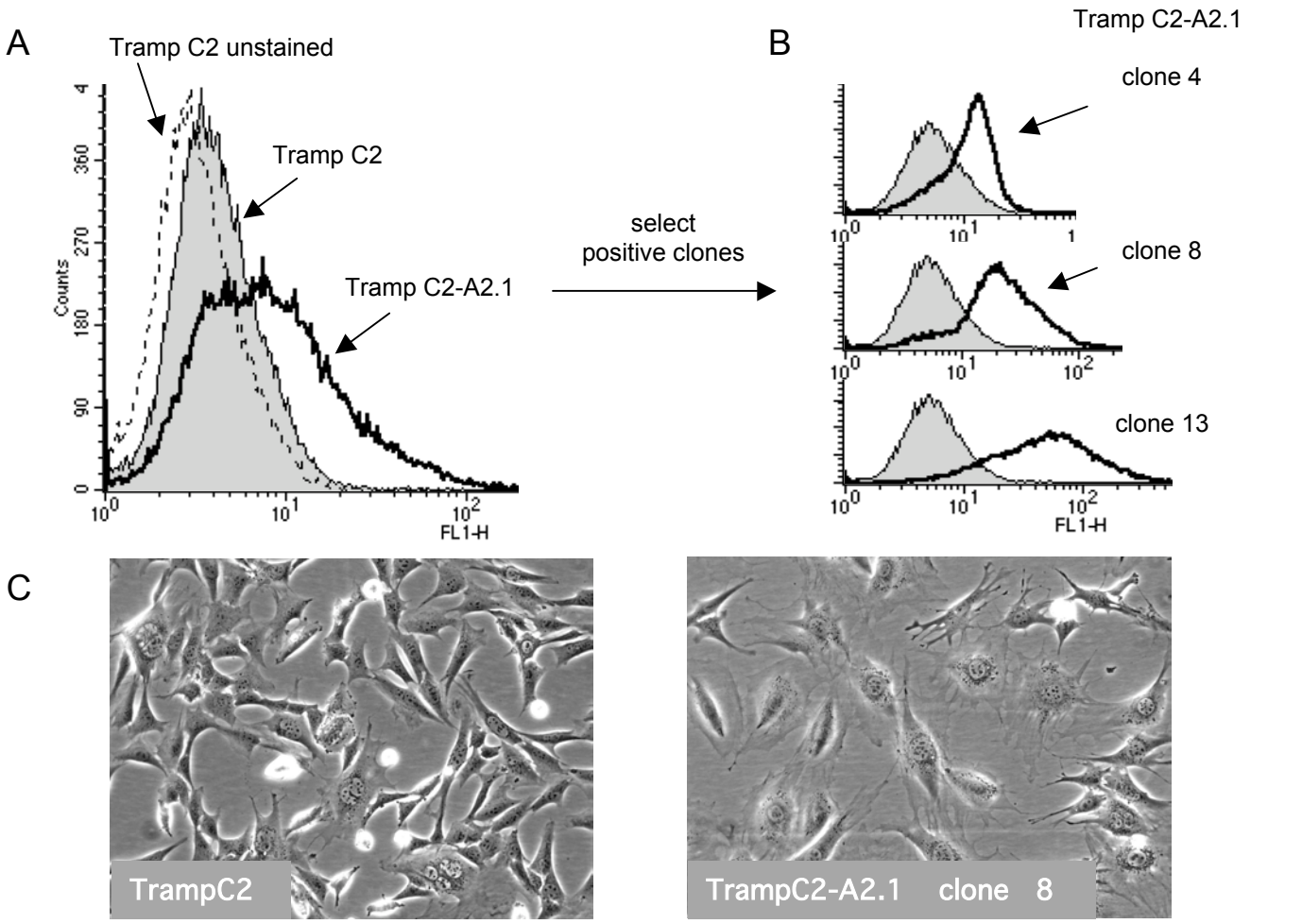

FIGURE 1: Viral transformation and clonal selection to establish stable TRAMP C2/2.1 cells. TRAMP C2 cells were transformed with a lenti virus carrying the human HLA2.1 gene. A) FACS analysis indicated that about 20% of treated cells are clearly HLA2.1 positive, which can be further improved by clonal selection (B). C) Virally transformed cells appear microscopically different (magnification x200).

stained clearly positive, clone 13 with the highest level of expression (Figure 1B showing 3 of the 5 positive clones). Interestingly, cells from all of these clones appeared to be bigger or more spread than the parental Tramp C2 cells, at least initially (Figure 1C). Whether this has any significance in terms the viability, growth rate and HLA2.1 expression remains unclear, but was also observed in virus-treated Tramp C1 cells (see below).

Tramp C1 cells were also treated with the HLA2.1 lentivirus as above. In an attempt to improve the selection of TRAMP C1 cells that are HLA2.1positive, cells were stained 5 days post transduction with the anti-HLA-A2 antibody and then sorted through magnetic separation using the MACS system (Miltenyi Biotec Inc.). After one week in culture, only 10% of sorted TRAMP C1/2.1 cells were HLA2.1 positive which prompted us to clonally

select those positive cells as above. 6 out of 30 clones had high levels of HLA2.1 expression (not shown). Again, HLA2.1 positive cells appeared bigger, more spread, and tended to grow slower in culture.

We then aimed to establish the tumorigenic potential of these HLA2.1 positive clones in vivo. All positive clones of TRAMP C1/2.1 and TRAMP C2/2.1 were injected into transgenic humanized C57BL/6 (C57Bl/6-K^{b}2.1) mice that express a chimeric class I molecule composed of the HLA-A2.1 extracellular domain while the transmembrane and intracellular parts remain murine H-2K^b (4). 1x10^6 cells were injected s.c., however none of the clones formed tumors. In comparison, parental TRAMP C2 tumors were palpable after 4 weeks in C57BL/6 mice, while TRAMP C1 tumors tended to form tumors even faster. We subsequently increased the inoculums up to 5x10^6 cells without success. Lympho-suppressive preconditioning with 5.5-6Gy sublethal whole-body irradiation prior to tumor injection to enhance tumor take was also fruitless. Similarly, cell suspensions within Matrigel and/or irradiated parental TRAMP C1 tumor cells as feeder layer alongside TRAMP C1/2.1 also failed. More than 100 mice have been injected over the course of 8 months. This is certainly surprising since Drs Koya and Ribas have used this virus extensively in lymphoma and melanoma cell lines with high efficiency and have not experienced any problems regarding tumor formation in the very same transgenic humanized C57Bl/6-K^{b}2.1 mice and we have performed similar studies successfully using B16 melanoma. The fact that TRAMP C2/2.1 did form tumors in 4 out of 4 injected SCID/beige mice suggests to us that immune rejection might play a role. We harvested those tumors and re-injected them as smaller pieces into C57Bl/6-K^{b}2.1 mice and we are currently awaiting the outcome.

We have ruled out the HLA status in C57Bl/6-K^{b}2.1 mice (Figure 2a) as being a factor. In fact, the transgenic mice express both human HLA2.1 and murine H-2K^b while C57Bl/6 have the mouse MHC class I, only (Figure 2b).

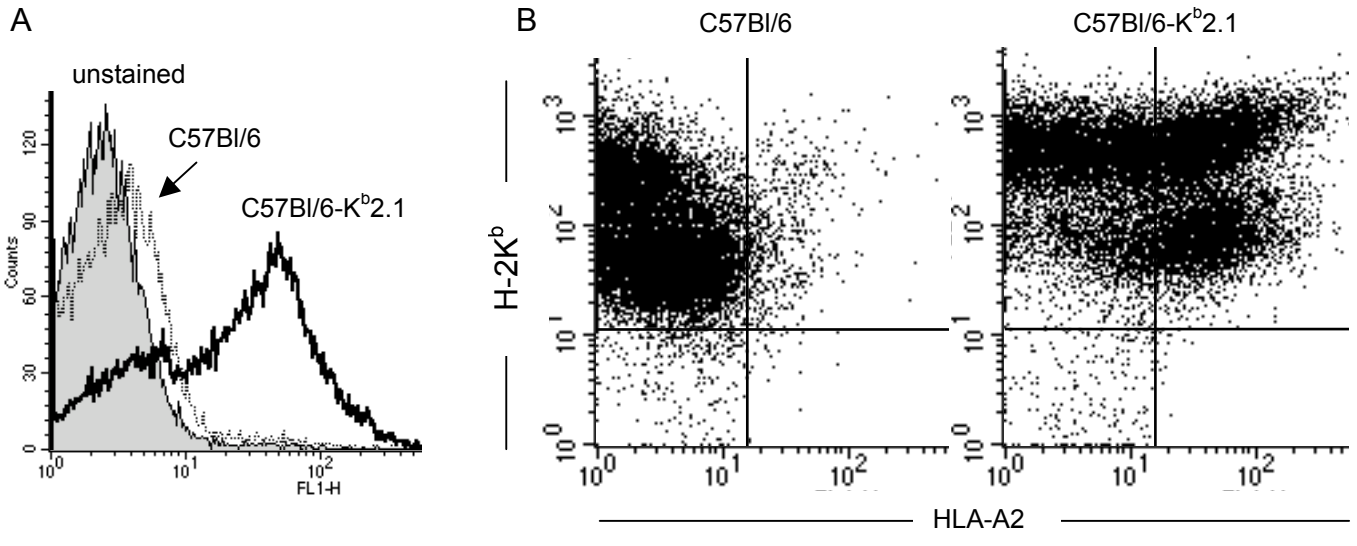

FIGURE 2: MHC class I expression in splenocytes from C57Bl/6-K^{b}2.1 transgenic mice. A) C57Bl/6-K^{b}2.1 are HLA2.1 positive while C57Bl/6 are not. B) Those splenocytes that are HLA2.1 positive (45%) are also positive for the mouse MHC I molecule (upper right quadrant). There are 2 distinct populations of spleen cells in either mouse strain having low or high H-2K^b levels, respectively.

We also checked the stability of HLA2.1 expression in individual clones. We noticed that some but not all clones maintain long-term expression; only those long-term positive clones were used for in vivo studies (Figure 3). Our next approach is to return to our original TRAMP C1/2.1 and TRAMP C2/2.1 cells (uncloned population) and inject those into transgenic animals. If the TRAMP-2.1 cells fail to form tumors in these humanized mice will switch to the standard TRAMP-C57Bl/6 system to fulfill our experimental aims.

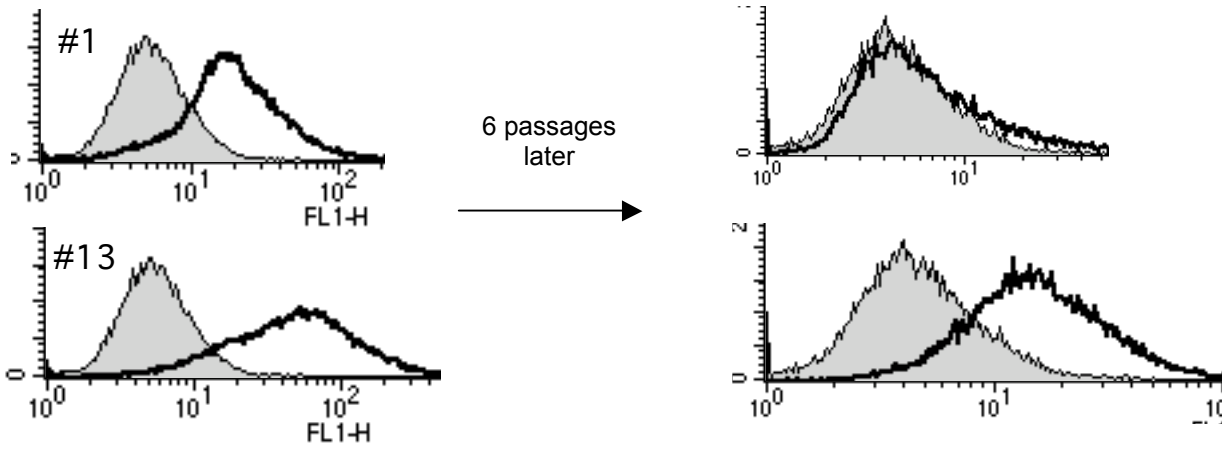

FIGURE 3: Not all TRAMP C2/2.1 clones maintain long-term HAL2.1 expression. HLA2.1 expression was assessed by FACS analysis. Clone 1 (top) lost HLA2.1 expression during in vitro culture while clone 13 (bottom) remained positive even after 18 passages (not shown).

As part of the fulfillment of Task 1 in our proposal we addressed the issue of survivin expression in vitro. There are indications that TRAMP tumor cells express survivin (5). We were able to confirm that this is the case for our TRAMP C cells and their HLA2.1 derivatives (Figure 4), which showed strong expression.
Once the TRAMP C1/2.1 and TRAMP C2/2.1 tumors grow in vivo we will verify survivin expression using the same techniques to ensure that there is no in vivo down-regulation of antigen expression. Normal C57Bl/6-K^b2.1 prostate tissue will serve as a control.

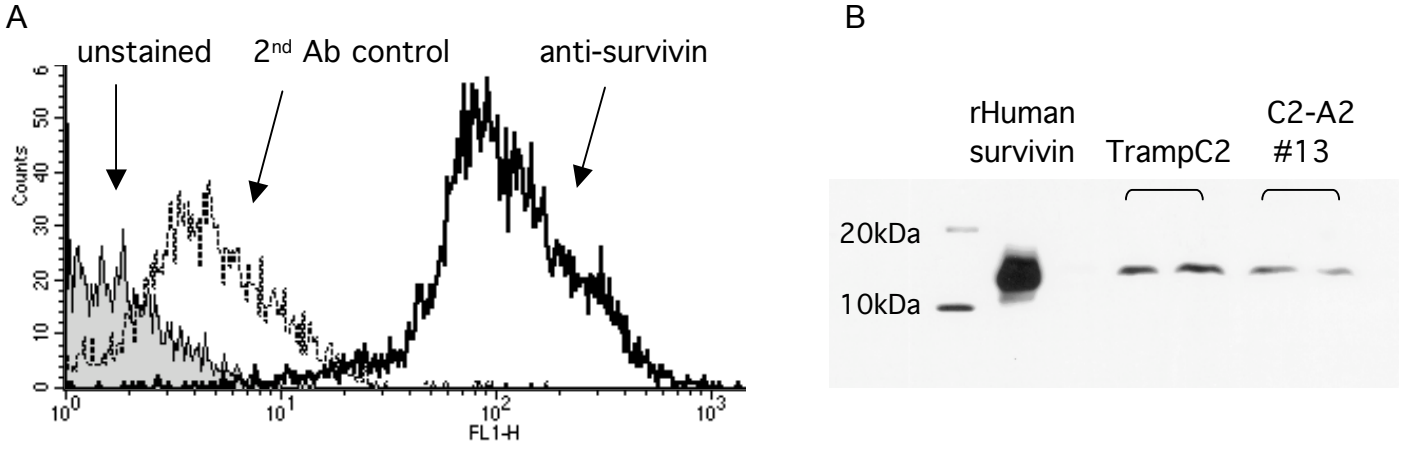

FIGURE 4: Survivin expression in TRAMP C cells in vitro. A) Intracellular staining of fixed and permeabilized TRAMP C2/2.1 cells, clone 13. (1st antibody: rabbit anti human/mouse survivin; Cell Signalling, 2nd antibody FITC-goat anti rabbit, BD). B) Western blot of whole cell lysates (not quantitative). Recombinant human survivin (R&D) served as positive control. Survivin runs at 16kDa.

The third and clinical task of our proposal aims to evaluate immune responses to tumor antigens in prostate cancer patients undergoing RT and to find out whether or not they are amplified by RT. We received ficoll-gradient isolated peripheral blood mononuclear cells (PBMC) from 13 patients with prostate cancer before, during and after they had undergone radiation therapy (Dr. Haustermans, University Hospital Leuven, Gasthuisberg, Belgium. We confirmed the HLA-A2 haplotype in 10 of these patients (HLA-A2 antibody clone BB7.2, BD Pharmingen) and these patients were therefore eligible for tetramer analysis of survivin-specific $CD8^+$ T lymphocytes that have the T cell receptor for the survivin peptide Sur1M2 (LMLGEFLKL, Beckman Coulter) (28 samples). Sample volume permitting a negative tetramer determined background PE fluorescence. Ficoll-Paque™-isolated PBMCs from a single volunteer was internal control for each assay. 1-2x10^5 events were accumulated. The gating strategy was (Figure 5):

1) plot FL3 , set viability gate (gate 1); (fixed cells served as control)
2) plot FL1 vs. FSC of population in gate 1; set gate 2 for $CD8^{high}$ lymphocytes, excluding NK cells ($CD8^{low}$)
3) plot FL-2 vs. FL-1 of cells in gate 1 and 2 (viable $CD8^{high}$ lymphocytes). Samples of the healthy volunteer stained with negative tetramer were used to set an arbitrary FL-2 lower limit of 0.03% double positives (6).

Quality control required ≥10,000 viable events and ≥2,000 $CD8^+$ T cells. However, one sample did not meet the minimum standards of no less than 10,000 viable events with at least 2,000 $CD8^+$ T cells and was therefore excluded. Sufficient material from 7 patients was available for tetramer analysis at all 3 time points, with 3 being tested at 2 time points,. Overall there were 9 samples before RT, 10 samples during RT and 8 samples after RT (Table 1).

In most cases, staining of $CD8^+$ cells with the survivin tetramer was significantly higher than with the irrelevant, negative control tetramer indicating the presence of antigen-specific T cells (Figure 6, p=0.002).

The HLA-A*0201^+ healthy volunteer had 0.11% ±0.04 of $CD8^+$ T cells reactive. First, we determined whether or not a sample was positive for survivin-specific T cells. The low-limit of detection (LLD) was taken to be the mean ± 2SD of the values for th9 healthy volunteer, i.e. 0.19%. By this criterion, 6 of 9 (66.7%) patients before treatment, 4 of 10 (40%) patients during treatment, and 6 of 8 (75%) patients after treatment were positive (Table 1). There appeared to be a trend towards an initial drop during RT with increased responses upon completion of therapy.

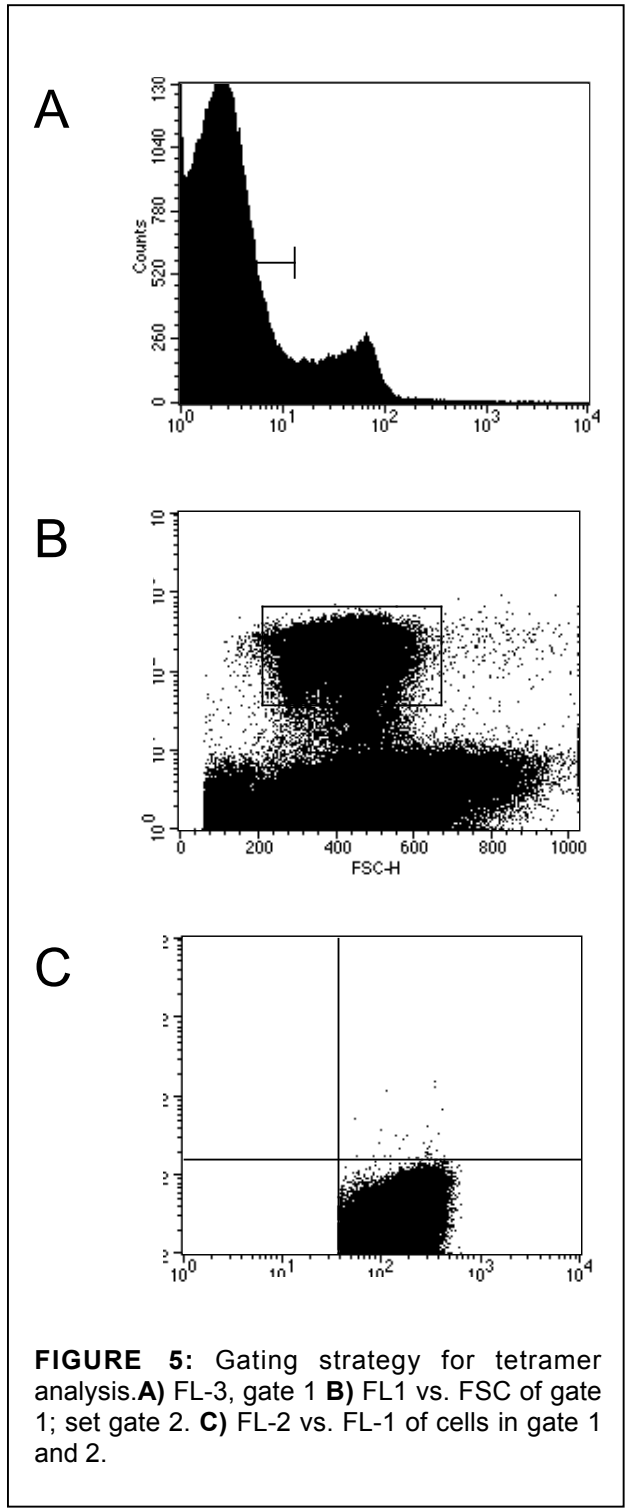

FIGURE 5: Gating strategy for tetramer analysis.**A)** FL-3, gate 1 **B)** FL1 vs. FSC of gate 1; set gate 2. **C)** FL-2 vs. FL-1 of cells in gate 1 and 2.

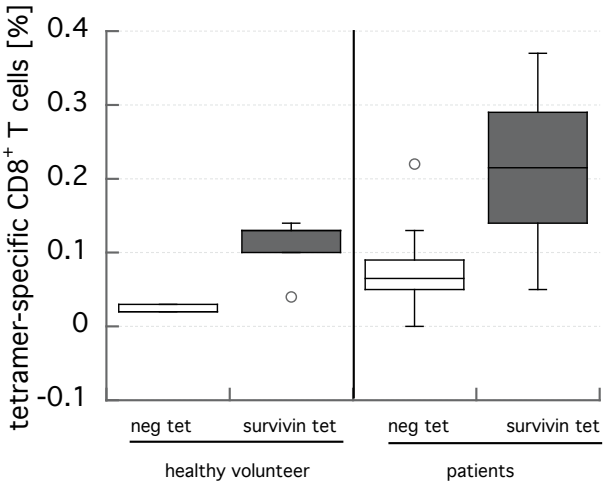

FIGURE 6: Specificity of the survivin tetramer. Human samples stain relative higher with the tetramer for survivin when compared to staining with the irrelevant, negative tetramer ($p<0.005$). Data are percent $CD8^+$ T cells staining positive with the tetramer for survivin or the negative tetramer, respectively.

TABLE 1: Increasing percent of cancer patients show survivin-reactive CD8+ T cells in peripheral blood upon completion of radiation treatment. Data are Data are CD8+ T cells staining positive with the tetramer for survivin [%] from 10 HLA-A2 positive patients with prostate cancer. Gray fields highlight those samples that stained above the background level (LLD = mean ± 2x SD of % survivin-reactive CD8+ T cells in the healthy volunteer).

patient	radiotherapy		
	before	during	after
prostate 1	0.23	0.29	0.37
prostate 2	0.12	0.05	0.25
prostate 3	0.28	0.23	0.53
prostate 4	1.33	0.16	x
prostate 5	0.22	0.12	x
prostate 6	0.15	0.15	0.26
prostate 9	0.46	0.16	0.15
prostate 11	0.32	0.31	0.21
prostate 12	0.15	0.5	0.32
prostate 13	x	0.1	0.14

Obviously, the number of samples is small but the power of the study is in its longitudinal nature. When patient responses were examined individually over time the tendency of circulating tumor-specific T cells to decrease during RT became very evident (Figure 7B). The number of survivin-specific $CD8^+$ T cells decreased in 66.7% patients (66.7%) during the course of radiotherapy (from *before* → *during*). Intriguingly, the majority of patients (5 out of 7 patients) ended up with more survivin-specific $CD8^+$ T cells in their circulation than before they had started treatment (*before* → *after* RT). This is very preliminary data and will have to be evaluated further. We are hoping to extend this study group to more than 30 patients. Furthermore, efforts are currently under way to determine the level of circulating T regulatory cells in these prostate patients on the basis of CD4, CD25 and FoxP3 positive staining (as outlined in Task 3.5). This will allow us to get an idea regarding immune suppression in these patients.

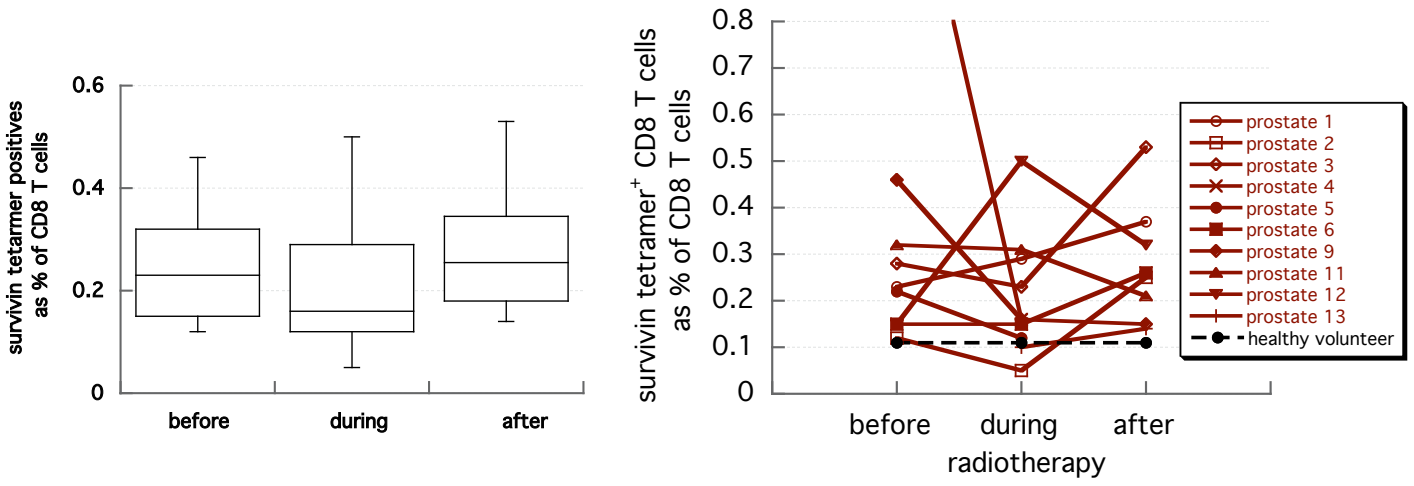

FIGURE 7: Levels of circulating survivin-reactive $CD8^+$ T cells initially drops during the course of radiotherapy but increases upon completion of radiotherapy. Data are shown percent $CD8^+$ T cells staining positive with the tetramer for survivin of **A)** median data in box plot or **B)** as time courses for each individual patient.

3. KEY RESEARCH ACCOMPLISHMENTS

- Generation of stable TRAMP C1 and TRAMP C2 mouse prostate cell lines, that express human HLA-A2.1 (TRAMP C1/2.1 and TRAMP C2/2.1). (in collaboration with Drs. Koya and Ribas, UCLA)
- Confirmation of HLA-A2 expression in TRAMPC/2.1 cells after transduction by antibody staining and FACS analysis.
- Isolation of individual TRAMP C1/2.1 and TRAMP C2/2.1 clones that maintain long-term expression.
- Successful growth of TRAMP C2/2.1 clone 13 in SCID/beige mice.
- Confirmation of survivin expression in vitro in parental TRAMP C2 and in their HLA2.1 derivatives using Flow cytometry and Western blotting.
- Isolation of peripheral blood mononuclear cells (PBMC) from blood samples from 13 patients with prostate cancer before, during and after they had undergone radiation therapy and from 1 healthy control (in collaboration with Dr. Haustermans, Leuven, Belgium).
- Confirmation of the MHC haplotype in 10 patients with a HLA-A2 specific antibody, and subsequent FACS analysis.
- Enumeration of circulating tumor-specific $CD8^+$ T lymphocytes in HLA-A2 positive prostate cancer patients using tetramers specifically binding T cells that have the T cell receptor for the survivin peptide Sur1M2 (LMLGEFLKL, Beckman Coulter). Preliminary data suggest that there are radiation therapy-related changes in this patient population.

4. REPORTABLE OUTCOMES

- Development of mouse prostate cancer cell lines TRAMP C1/2.1 and TRAMP C2/2.1, that stably express the human MHC I molecule HLA-A2.1.
- **D. Schaue**, Y. Liao, E. L. Kachikwu, B. Comin-Anduix, A. Ribas, L. Goodglick, A. Debucquoy, K. Haustermans, and W. H. McBride: Human T Cell Responses to Survivin in Cancer Patients Undergoing Radiation Therapy. In: Proceedings of the American Association for Cancer Research in Los Angeles, CA, 2007.
- Kachikwu, E., **Schaue, D**., Liao, Y-P., Iwamoto, K.S., Economou, J.S., and McBride, W.H.: The Role of Naturally-Occurring T Regulatory Cells in Radiation-Induced Immune Modulation in Murine Prostate Cancer. Manuscript in preparation.

5. CONCLUSION

The first aim of this proposal is to determine as to whether local irradiation of prostate tumors in mice alters measurable tumor-specific immune responses. The preclinical studies in this proposal use TRAMP C1 and TRAMP C2 mouse prostate cell lines, that express human HLA-A2.1 (TRAMP C/2.1) and grow them s.c. in transgenic humanized C57BL/6, which will allow us to examine the responses to human survivin epitopes that are clinically relevant.

In summary, we were able to generate stable TRAMP C1 and TRAMP C2 cell lines that express the human MHC class I molecule HLA2.1. using a recently developed Lentivirus. This is an important step, firstly because TRAMP C1 and TRAMP C2 are generally known to be difficult to transfect and secondly because this will allow us to examine the responses to human antigenic epitopes (in our case: survivin) that are clinically relevant in our humanized mouse model (C57Bl/6-K^b2.1), that express the chimeric mouse/human class I MHC. The fact that these transfected cell lines form tumors in SCID/beige mice but not in C57Bl/6-K^b2.1 is interesting and challenging. It suggests to us that they have maintained their tumorigenic potential but that immune-mediated mechanisms might be in place that prevents tumor formation in C57Bl/6-K^b2.1 mice. Ultimately, in the interest of time, if none of these cell lines and/or clones form tumors we will return to the parental cell lines and

investigate the efficacy of combination therapies to target prostate cancer in the original C57Bl/6 mice, keeping in mind that ways of immune monitoring will be more limited in that system.

We were clearly able to detect survivin-reactive T cells by tetramer binding in more than half of prostate patients when compared to binding of a negative tetramer or to our healthy control. This is in agreement with others (7, 8) and it suggests that anti-survivin responses do indeed occur in cancer patients. This is important because although reverse immunology has clearly established that survivin is immunogenic and that survivin-specific responses can be elicited in vitro, it had been uncertain whether anti-survivin responses occur in vivo in untreated cancer patients. More importantly, we were able to show that the level of these tumor-specific T cells changed as patients underwent radiation treatment, initially declining but eventually rising so that most patients had more of these T cells in their circulation after completion of RT. Ultimately, whether this immune response is associated with tumor down-staging awaits further analysis, since this trial is still under way.

It is tempting to ascribe the increase in tumor-specific T cells to a radiation-induced increase in antigenic peptide liberation and dendritic cell activation (9, 10), and we have shown that presentation of exogenous antigen by dendritic cells can be boosted by radiation (11). Among possible mechanisms by which RT might stimulate immune responses are radiation-induced up-regulation of molecules such as Fas, MHC I and II and co-stimulatory CD80 molecules on tumor and dendritic cell surfaces that can enhance both immunogenicity and susceptibility to T cell-mediated attack (12-14), improved T cell trafficking (15), and altered proteasome expression/function that might modify the antigens presented (16). Further work is necessary to elucidate which of these might operate functionally in a clinical setting and to answer the all-important question as to whether the anti-survivin response that is generated by RT assists tumor regression. What is clear is that RT does not induce immune tolerance to survivin making IT approaches feasible in combination with RT, as has also been suggested from the recent clinical vaccination trial in prostate cancer (17).

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7. APPENDIX

N/A

8. SUPPORTING DATA

N/A